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Temperature-Independent Period Immediately After Fertilization in Sea Urchin Eggs

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Abstract. The experiments described here explore the regulation of the duration of cleavage cycles in sea urchin eggs. We measured the timing of early cleavages in individual fertilized eggs under various temperature conditions and applied methods of statistical analysis to the data obtained. At least for the first three cleavages, the temperature dependence of the cleavage intervals was nearly equal. In addition, we identified a temperature-independent period at the beginning of the first cleavage cycle. This period occurs immediately after fertilization and lasts for several minutes. Our results suggest that this interval of temperature independence is related to the process of egg activation.

Introduction

Various studies have been conducted on the mechanisms that regulate the cell cycle at the molecular level, *e.g.*, the cyclic accumulation and destruction of cyclins and the cyclic activity of M phase-promoting factor (MPF) (Meijer *et al.*, 1991; Edgecombe *et al.*, 1991). Such studies have used conventional methods that determine the timing of an event in the cell cycle by measuring some condition or activity in a whole group of cells at given time intervals—for example, by plotting the percentage of cells that had reached a certain stage or the amount of a particular biochemical activity over time.

However, such methods have limited value because the average behavior of a group of cells reveals little about individual cells. Questions such as how much fluctuation is experienced by individual cells in the duration of interphase periods cannot be answered. Thus it is difficult to apply methods of statistical analysis, including the so-called method of moments, confidence interval determination, and regression analysis, especially to the cleavage intervals.

If cell cycle events differ greatly in their degree of temperature dependence, we should be able to use those differences to detect the events. Therefore, we attempted to measure the timing of several early cleavages in sea urchin eggs of various temperatures. These eggs are arrested in post-meiotic interphase and undergo a series of rapid and highly synchronous cleavages after fertilization.

We developed a system by which we can determine the length of several early cleavage cycles in sea urchin eggs by timing these cleavages in tens of individual eggs. Using this system, we found that the effect of temperature on the duration of the cleavage cycle is approximately equal for successive cycles. However, by applying methods of statistical analysis, we also discovered a temperature-independent period of several minutes immediately after fertilization, which has not been found previously (Hoadley and Brill, 1937; Matsumoto *et al.*, 1988).

Materials and Methods

Handling of eggs

Gametes of the sea urchins *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus* were obtained by injection of 1 m*M* acetylcholine chloride into the body cavity. Semen was collected "dry" and stored at 4°C until use. The eggs were washed several times with artificial seawater (ASW) and stored as a gently stirred suspension at 5°–10°C. In each experiment, gametes obtained from the same parents were used throughout.

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Time-lapse video microscopy

The time course of early cleavage was recorded for groups of fertilized eggs (about 30 in each group). The eggs were photographed with a CCD camera (Hamamatsu, C2400) that was mounted on an inverted phase-contrast microscope and attached to a videocassette recorder (Sony, EVT-820).

While the fertilized eggs were being videotaped, it was necessary to regulate the temperature of the ASW that flowed through the observation vessel. For this purpose, a homemade thermo-control chamber was positioned on the stage of the microscope and the observation vessel—a glass laboratory dish, which was filled with 1.5 ml of ASW—was placed into it. The control chamber, which was made of acrylic resin, held water to a depth less than the height of the glass dish. A ring-shaped copper pipe that was immersed in the water of the chamber encircled the glass dish and circulated water from a thermostat bath. The temperature of the ASW in the dish was kept constant by passing it through a tube coiled around the pipe before it entered the dish. (The difference in temperature among locations in the field of the microscope was at most 0.1°C.) To prevent the eggs from being flushed out of the glass dish during observation, they were placed on a nylon mesh that was fixed to the bottom of the dish with wax. A sensor set next to the mesh confirmed that the temperature of the ASW was stable to within 0.1°C throughout each observation.

In all except the temperature-jump experiments described below, gametes were inseminated and after 1 min were added to the glass dish in the thermo-control chamber. After the observation period, the videotape was played back and forth so that the time interval between insemination and the beginning of first cleavage could be determined for each egg. Cleavage of the plural blastomeres of each egg was nearly synchronous in the second and third cycles, so these cleavages were judged from a single blastomere. In this manner the time intervals up to the third cleavage were determined for individual eggs. The length of the first cleavage cycle (u_1) was defined as the time interval between insemination and first cleavage. The length of the second cleavage cycle (u_2) was determined by subtracting the time of the first cleavage from the time of the second, and so on.

The temperature-jump experiments required two observation setups. A sample of fertilized eggs was suspended in a test tube in a 9.6°C constant-temperature water bath. At each predetermined time after insemination, a drop of the egg suspension was added to a glass observation dish that had been set at 17.3°C in one thermo-control system. This constituted the "jump" in temperature. (Measured temperature recovered from a fall in temperature to within 0.1°C at about 1 min after addition of the eggs.) As a control, another sample of the fertilized eggs was dropped into an observation dish, which had also been set at 17.3°C, in the second thermo-control system, at 1 min after insemination. Cleavage cycles for the eggs in the temperature-jump experiments were determined as previously described.

Results

Temperature dependence of cleavage cycles

Figure 1 shows the reciprocal of the length of the cleavage cycles (*i.e.*, cleavage rates) in *P. depressus* eggs. This plot is analogous to an Arrhenius plot, with log rate ($\ln(1/\langle u_i \rangle)$) as a function of the reciprocal of absolute temperature; where $\langle u_i \rangle$ is the mean for the length of the i-th cleavage cycles, as defined in the Materials and Methods. Plotted in this manner, the three curves run parallel to one another (with the curve for u_1 shifted along the ordinate axis from the two other curves). This suggests that the temperature dependence of the three cleavage cycles is similar. The rationale follows.

Because temperature dependence is a change in relative rate (compared with the rate at one standard temperature)

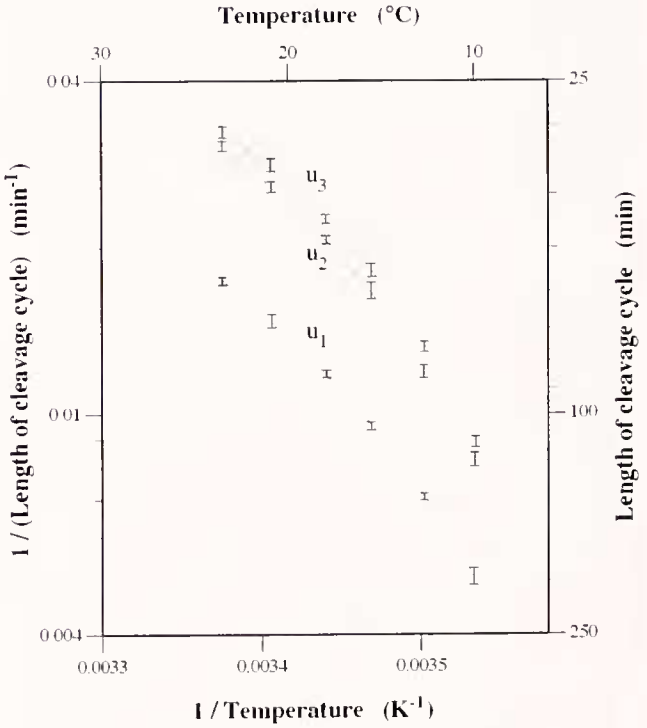

Figure 1. Temperature dependence of the length of cleavage cycles. Each set of the reciprocals of the averages for the first three cleavage cycles (u_1, u_2, and u_3) in *Pseudocentrotus depressus* eggs is plotted against the temperature at which each sample was kept. The number of eggs in each sample is about 30. Error bars show 99% confidence intervals of the averages.

with temperature, each cleavage rate is represented by the product of the temperature-dependent term and the cleavage rate at a standard temperature, which is a constant that differs between cleavages. The logarithm of this product is thus the sum of the logarithm of the term representing the temperature dependence and the logarithm of the constant. So, if all terms of the temperature dependence are equal to one another from one cleavage cycle to the next, all curves as a function of temperature will have the same shape with different ordinate intercepts.

To further examine the temperature dependence of cleavage cycles, the same data used in Figure 1 are replotted in Figure 2. As seen in the figure, u_3 has a linear relation to u_2, with the straight line passing through the origin; its slope is 0.9. The ordinate intercept is not significantly different from zero ($P = 0.16$). This relation shows that the temperature dependence of u_2 is equal to that of u_3, since the lengths of two cleavage cycles should be in direct proportion to one another only if the temperature dependence of the cycles is unchanged from one to the other.

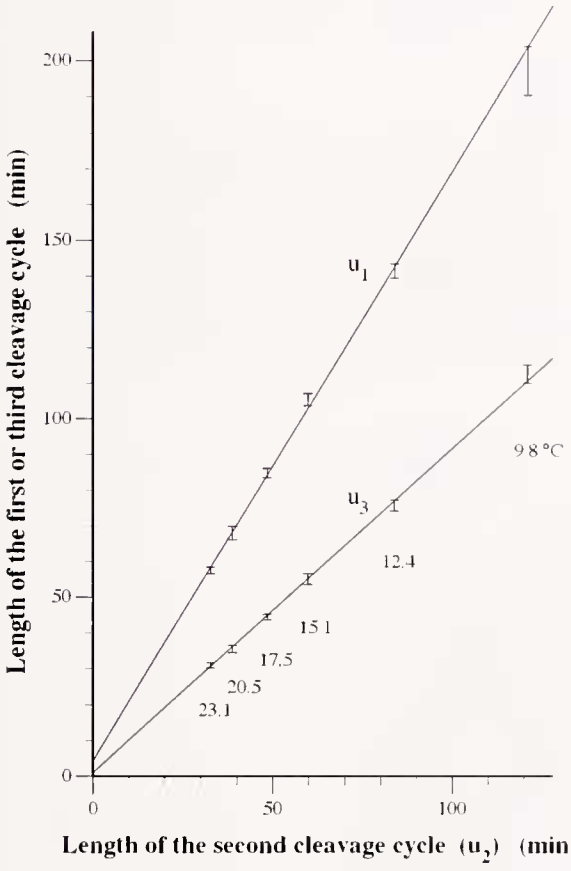

Figure 2. The data in Figure 1 plotted to show the relation between cleavage cycles at various temperatures. The abscissa is the mean length of the second cleavage cycle, and the ordinate is the mean length of the first or third cleavage cycles. Error bars show 99% confidence intervals of the averages. The error bars for u_2 were omitted for clarity in this figure (the magnitude of error bars for u_2 is similar to the magnitude of error bars for u_3). Each straight line was obtained by at least-squares method applied to the data points for u_1 or u_3, using the squares of SEM for both variables as the variances (Awaya, 1983). The ordinate intercept of the line is 4.1 min for u_1 and 0.8 min for u_3.

Cycle u_1 also has a linear relation with u_2. However, the ordinate intercept for this straight line is 4.1 min and significantly non-zero ($P < 0.001$). Its slope is not 1 but 1.6, corresponding to the vertical shift of the curve for u_1 from the two other curves. That is, in extrapolating to a hypothetical condition in which u_2 is zero (corresponding to a rate of cleavage that has been raised to infinity by imaginary heating), u_1 does not become zero but has a limited value. Therefore, u_1 appears to include a temperature-independent period of several minutes that does not exist in u_2 or in u_3.

Temperature-jump experiment

To determine when the temperature-independent period occurs within the first cleavage cycle, we used temperature-jump experiments. Fertilized eggs of *H. pulcherrimus* were kept under constant low temperature (9.6°C) for various incubation times, then placed into ASW at a higher temperature (17.3°C); the total length of the first cleavage cycle was measured for each cell. Because a temperature-independent term should remain constant when temperature changes, whereas a temperature-dependent term will get longer when temperature falls and shorter when it rises, we can distinguish the temperature-independent term from the temperature-dependent term.

Figure 3 shows the results of nine pair of measurements—that is, control and experimental values for 9 incubation times. The average of the duration times measured for the first cleavage cycle for the temperature-jump sample was subtracted from the control value, and these differences were plotted to show cycle delay as a function of incubation time. No delay in u_1 is observed when the incubation time is shorter than several minutes. Thereafter the length of the first cleavage cycle increases linearly with the incubation time. An abscissa intercept given by the linear least-squares method, to which all data pairs were subjected, was significantly different from zero ($P < 0.001$). An abscissa intercept was 5.2 min when the linear least-squares method was applied to all data pairs except the one for the shortest incubation time. This shows that a temperature-independent period of several minutes exists immediately after fertilization. (Note that if a temperature-independent term appeared later in the first cleavage cycle, a corresponding horizontal segment of the plotted data would appear further to the right than at the beginning of the ascending line.)

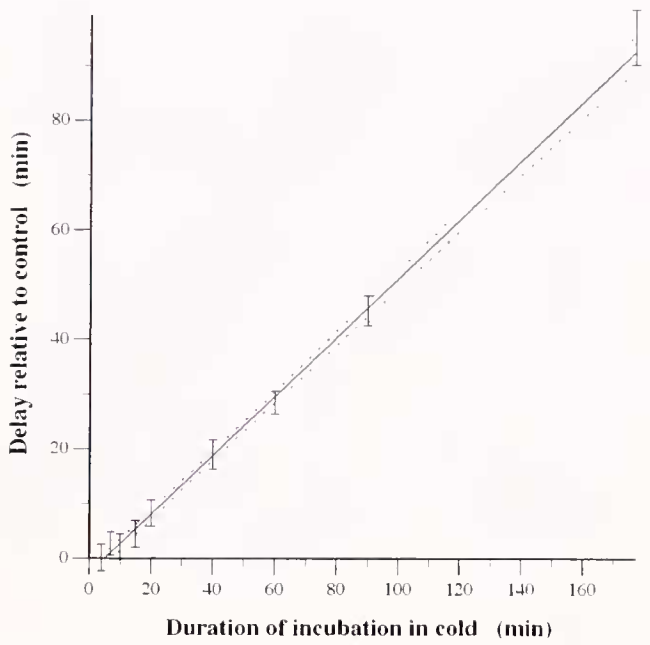

Figure 3. Temperature-jump experiment showing the delay in u_1 relative to control in *Hemicentrotus pulcherrimus* eggs as a function of the duration of incubation at low temperature. Each error bar shows the 99% confidence interval of the difference between the average for u_1 in the temperature-jump sample and the average for u_1 in its control, at each of various incubation times (given in the abscissa). The data point for the longest incubation time is from the measurement in which eggs were incubated at 9.6°C throughout; its abscissa value is the mean length of the first cleavage cycle of the eggs. The straight solid line was obtained by applying least-squares fitting to all the data points except the one for the shortest incubation time, using the sum of the squares of the SE of two coupled averages as the variance. The broken lines give the endpoints of the 99% confidence interval for the true mean of the ordinate value for a given abscissa value (Green and Margerison, 1977). The 99% confidence interval for the abscissa intercept of the regression line, which is predicted from the above endpoints (Draper and Smith, 1981), is between 3.0 and 7.2 min.

Discussion

Temperature dependence of the processes affecting the length of cleavage cycles

Various processes, acting in sequence or in parallel, make up the cell cycle, but only some of these affect the length of the cycle (Nurse, 1990). The observation of the temperature-independent period immediately after fertilization means that there are one or more temperature-independent processes affecting the length of the first cleavage cycle.

The cleavage cycles differ in absolute length, and thus they must also differ in the ratio between the duration of the processes governing the length of the cleavage cycles and the total duration of the cycles. Nevertheless, as shown in the Arrhenius plot (Fig. 1), and the plot of u_1 *versus* u_2 or u_3 *versus* u_2 (Fig. 2), the cleavage cycles are very nearly equal in temperature dependence. Apparently, then, the specific processes that affect the length of cleavage cycles at a given temperature differ little from each other in temperature dependence, with the exception of the temperature-independent process or processes immediately after fertilization. This conclusion is supported by the high linearity of the plot for the temperature-jump experiment (Fig. 3; a value of $\chi^2 = 11$ for 6 degrees of freedom, corresponding to $P = 0.09$ in a χ^2 test for goodness of fit (Bevington and Robinson, 1992)).

Although we have classified the processes that affect the length of cleavage cycles as either independent of or dependent on temperature, the period immediately after fertilization is not absolutely temperature-independent, but only relatively by comparison with the later periods of the cycle. In reality, the first period may have a weak dependence on temperature that might cause the deviation from the linear relation in the plot of u_1 *versus* u_2 (Fig. 2).

Temperature-independent event associated with egg activation

Both the relationship between cleavage cycles (Fig. 2), whose durations are a function of temperature, and the results of the temperature-jump experiment (Fig. 3) showed that the first cleavage cycle contains a unique temperature-independent term that lasts for several minutes. In addition, the temperature-jump experiment demonstrated that this term takes place immediately after fertilization.

The first cleavage cycle is relatively long in comparison to the ones that follow. This is partly because egg activation, which triggers the resumption of the cell cycle of the egg immediately after fertilization, includes a large number of processes (reviewed by Whitaker and Steinhardt, 1985; Whitaker and Patel, 1990). Considering that the temperature-independent period is specific to the initiation of the cleavage cycles and is transient, this term probably originates in an event related to the activation of the egg rather than to the cell cycle *per se*. Since the temperature-independent term affects the length of the first cleavage cycle, this event is presumably important in egg activation. Furthermore, because it is not preceded by a temperature-dependent period, the temperature-independent event is probably the overriding change during egg activation, and the temperature-dependent events that follow presumably cannot take place until it occurs.

When a sea urchin egg is fertilized, the cytoplasmic concentration of calcium and hydrogen ions changes markedly. These ionic signals are the necessary and sufficient stimuli for the activation of protein synthesis, DNA synthesis, and other metabolic processes that constitute egg activation (Whitaker and Steinhardt, 1985). In particular, cytoplasmic alkalinization, which is mediated by the

activation of a Na^+-H^+ exchange (Johnson *et al.*, 1976) similar to the mechanism that functions in cultured cells (Payan *et al.*, 1983), is the major immediate cause of the increase in the synthesis rate of these macromolecules. The length of the temperature-independent period we have observed coincides with the increase in intracellular pH, which begins 1 minute after fertilization and lasts for several minutes (Whitaker and Steinhardt, 1985). In addition, the rate of Na^+-H^+ exchange is relatively insensitive to temperature in cells of the opossum kidney (Graber *et al.*, 1992). This period of cytoplasmic alkalinization may correspond to the temperature-independent period.

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